Reprinted from
JOURNAL OF APICULTURAL RESEARCH
A journal of the Bee Research Association

SEPARATION AND IDENTIFICATION OF SOME ORGANIC PHOSPHATES IN HONEY BY COLUMN AND PAPER CHROMATOGRAPHY*

MARY H. SUBERS, ABNER I. SCHEPARTZ

Eastern Utilization Research and Development Division,
Agricultural Research Service,
United States Department of Agriculture
Philadelphia, Pennsylvania 19118, *U.S.A.*

and

ROBERT P. KOOB

Department of Chemistry
Saint Joseph's College
Philadelphia, Pennsylvania 19131, *U.S.A.*

Manuscript received for publication 20th February 1966

SUMMARY

Partial separation and tentative identification of the organic phosphates present in the phosphate fraction of honey have been accomplished by using a silica gel column and several paper chromatographic procedures. The organic phosphates identified were glucose 6-phosphate, 2- or 3-phosphoglyceric acid and α or β-glycerophosphate. Inorganic phosphate was also found. One organic phosphate was not identified.

INTRODUCTION

The existence of organic phosphates in honey is of interest because of their possible role as substrates or end products in the enzyme systems present.

Until 1958, the study of phosphorus in honey was confined to its presence as one of the minerals in honey ash. A review of early investigations, beginning with that of Erlenmeyer and von Planta in 1874, was given by Schuette and Huenink (1937) in their study of the influence of phosphorus, calcium and magnesium on honey colour.

White and Petty (1955), in their study of the organic acids in honey, found that there were several types of inorganic anions in the phosphate fraction. In a more detailed study of this fraction, Petty (1955) found both organic and inorganic phosphate.

The purpose of this investigation was to determine the nature of the organic phosphate by means of column and paper chromatography.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade and used as supplied. An unprocessed clover honey from Finger Lakes Honey Producers Co-operative †, Groton, N.Y. was used.

---

* From a thesis submitted by Mary H. Subers in partial fulfilment of the requirements for the Degree of Master of Science in Chemistry at Saint Joseph's College.

† Mention of a company or its products does not constitute endorsement by the Department of Agriculture over others not named.

The standard phosphates listed with abbreviations (if used in the text) and source are as follows: Fructose 1-phosphate, fructose 6-phosphate, ribose 5-phosphate, 6-phosphogluconate, adenosine 5′-triphosphate, nicotinamide-adenine dinucleotide, flavin mononucleotide, nicotinamide-adenine dinucleotide phosphate, uridine 5′-monophosphate, uridine 5′-disphosphate, 2,3-diphosphoglyceric acid (2,3-PGA), dihydroxyacetone phosphate (DHAP), glyceraldehyde 3-phosphate G-3-P), DL α-glycerophosphate (α-GP), DL β-glycerophosphate (β-GP), 2-phosphoglyceric acid (2-PGA), and phospho(enol)pyruvate (PEP) from Sigma Chem. Co., St. Louis, Missouri; glucose 1-phosphate (G-1-P), glucose 6-phosphate (G-6-P), phosphoserine and phosphoethanolamine from Calbiochem, Los Angeles, California; 3-phosphoglyceric acid (3-PGA), mannose 6-phosphate (Man 6-P), and phosphothreonine from Nutritional Biochem. Corp., Cleveland, Ohio; sodium phosphate tribasic dodecahydrate, J. T. Baker Chem. Co., Phillipsburg, N.J.; glucose, Nat. Bureau of Standards, Washington, D.C., Sample No. 41; phosphotyrosine, courtesy of Mr. Norbert Hipp and fructose 1,6-phosphate, courtesy of Dr. Jonathan W. White, Jr.

Apparatus

Three glass columns, two 64 × 2·5 cm. I.D. and one 64 × 4 cm. I.D., for ion exchange treatment of the honey.

One glass column, 35 × 1·2 cm. I.D., fitted with a Teflon stopcock regulated with a needle valve for silicic acid chromatography.

Four glass tanks, 61 cm. high × 30 cm. square, each containing four glass troughs arranged for descending chromatography on 21 × 56 - cm. papers.

Whatman No. 1 filter paper:

'Acid-Versene-washed paper' washed according to Runeckles and Krotkov (1957).

'Formic acid washed paper' washed in 1N formic acid only. In both methods the paper was washed in rectangular, Büchner-type Plexiglas filter.

General Electric 15-watt germicidal ultraviolet lamp to develop the blue phosphomolybdate spots (Bandurski & Axelrod, 1951).

Museum jar 26 cm. high, 26 cm. deep, 13 cm. wide containing a glass trough in the top and bottom for elution of phosphate areas from papers.

Preparation of the phosphate fractions

Honey (500 g.), diluted to 20% solid material, was put through ion exchange columns according to a modification of the procedure of Stinson *et al.* (1960). The phosphate material, which was retained on the Duolite A-4 (OH^-) column (instead of Duolite A-2 used by Stinson *et al.*), was stripped from this column with 1N NaOH and then passed through a larger Dowex 50 (H^+) column (350 c.c. bed vol.) to remove excess NaOH. This acid phosphate solution was partially lyophilized to reduce its volume, and an insoluble, phosphate-free precipitate which appeared during this concentration was filtered off and discarded. The concen-

trated solution was then lyophilized completely and redissolved in 3·5 ml. of 0·01N HCl. Phosphate (8·4 mg.) was found in this concentrate by the method of Paul (1960). To eliminate phosphate-free organic acids, 3 ml. of the concentrate was passed through a silica gel column according to the procedure of Bulen *et al.* (1952) with the modification of Stinson *et al.* (1960). Fractions were eluted with batchwise additions of *n*-butanol-chloroform mixtures containing 35, 50 and 70% *n*-butanol according to the following schedule: 104 ml. 35% *n*-butanol, 296 ml. 50% *n*-butanol, 175 ml. 70% *n*-butanol. No phosphate was eluted with the 35% *n*-butanol-chloroform mixture, as shown by the absence of a blue spot when a drop of the eluate on a piece of filter paper was sprayed with molybdate reagent (Hanes & Isherwood, 1949). The eluates from the 50 and 70% *n*-butanol-chloroform mixtures were collected in 61 and 24 five-ml. fractions, respectively. The five-ml. fractions were individually titrated to *p*H 8·3 with 0·01N NaOH and grouped in consecutive order (Table 1) into larger fractions according to their

TABLE 1. Description of fractions eluted from the silica gel column

Fraction no.	*Total volume (ml.)*	*Eluate (% n-butanol)*	*Designation in text*
1 – 4 inclusive	20	35 – 50	—
5 – 9	25	50	—
10 – 38	145	50	Fraction 1
39 – 59	105	50	Fraction 2
60 – 72	65	50 – 70	Fraction 3
73 – 87	75	70	—

acidity. This grouping was somewhat arbitrary, since a well defined curve was not obtained when the volume of NaOH required for each fraction was plotted against the fraction number. A blue phosphomolybdate spot was produced by all six of these large fractions in the same procedure described for the 35% *n*-butanol eluate. All the fractions were lyophilized, redissolved in 0·1 – 0·2 ml. of distilled water, and converted to their ammonium salts by the method of Agarwal *et al.* (1963). The three large fractions that contained the most phosphate (fractions 1, 2, 3, Fig. 1, Table 1) and the ammonium salt of the original concentrate (fraction 0. Fig. 1) were examined by paper chromatography. The other three silica gel column fractions (Table 1) were not studied at this time. All fractions were stored at −15°C.

Preparative paper chromatography

A fraction (0·15 ml.) was applied as a 2 × 210 - mm. streak across the top of a 21 × 56 - cm. sheet and developed according to procedure A (Table 2).

Strips (2 cm. wide) were cut lengthwise from each side of the sheet and sprayed with the moylbdate reagent to locate the phosphate areas. Corresponding areas were cut from the unsprayed section of the paper, and phosphates were eluted with water in the humid atmosphere contained in the museum jar. Elution was continued until eluate droplets on a piece of filter paper showed no blue colour with the molybdate reagent. The eluate was treated with Dowex 50 (H^+)

TABLE 2. Paper chromatographic procedures

Designation in text	*Procedure*	*Solvent system*	*Paper treatment*	*Development time (hours)*	
A	Agarwal (1963)	*t*-butanol ammonia soln., water; 6:3:1 (29·9% NH_3, s.g. 0·896)	*A* acid-Versene washed	A	39·5
B	(Agarwal *et al.*, 1963)		*B*[c] borate-impregnated acid-Versene washed	B	39·5
C	Agarwal (1962) (Agarwal *et al.*, 1962)	*t*-butanol, 50% formic acid, water; 16:1:4	formic acid washed		39·5
D	Modified Agarwal-*a* (Subers *et al.*, 1965)	1[a] Albon-Gross (1952) system *n*-propanol, ethyl acetate, water; 7:1:2	formic acid washed	1	15·5
E	Modified Agarwal-*b* (Subers *et al.*, 1965)	2[b] Agarwal (1962) system 1[a] Albon-Gross (1952) system 2[b] Agarwal (1963) system	formic acid washed	2 1 2	20 15·5 20
F	Modified Agarwal-*c* (Subers *et al.*, 1965)	1[a] Albon-Gross (1952) system 2[b] Mortimer (1952) ammonia system methyl cellosolve, methyl ethyl ketone, 3N ammonium hydroxide; 7:2:3	formic acid washed	1 2	15·5 20

[a] Paper dried after development with system 1, then developed in the same direction with system 2.
[b] Solvent system was not permitted to run off paper.
[c] Paper dipped into a 0·002M sodium tetraborate solution and allowed to dry just before use.

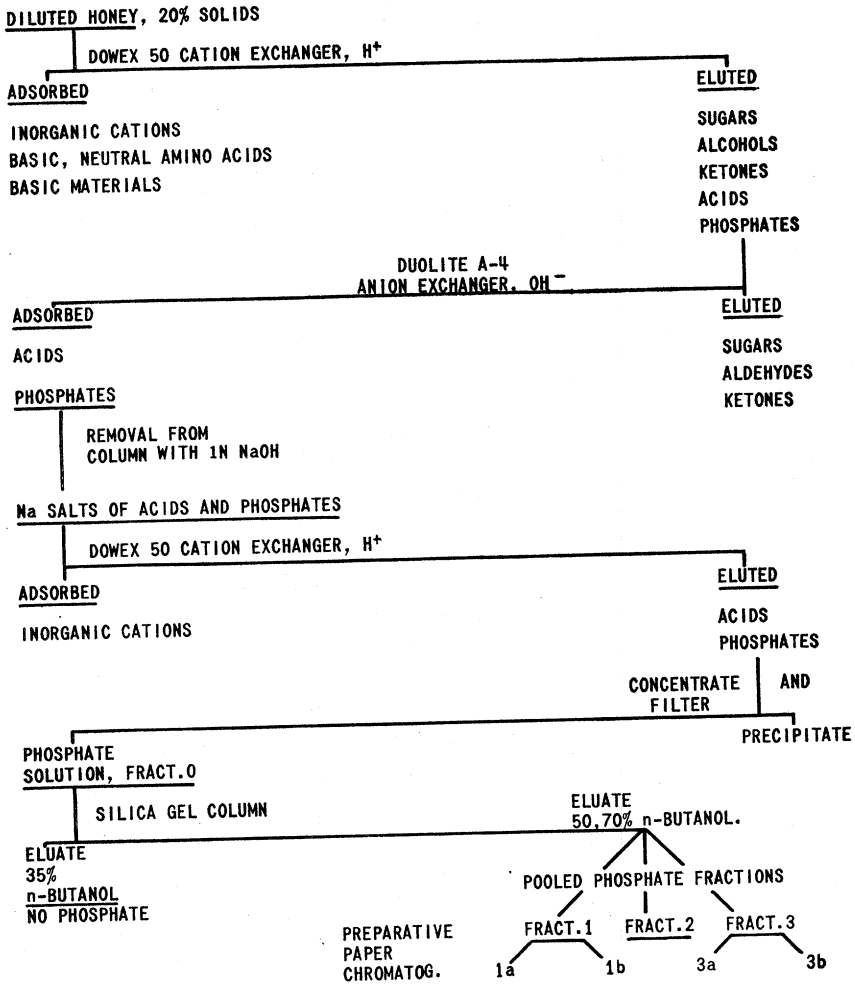

FIG. 1. Flow chart showing the derivation of the phosphate fractions from the honey

beads, converted to its ammonium salt according to Agarwal *et al.* (1963), lyophilized and redissolved in 0·2 – 0·3 ml. of distilled water.

Paper chromatographic procedures

Descending chromatography at 25 – 30°C. on 21 × 56 cm. sheets of washed Whatman No. 1 paper was performed in all the procedures described in Table 2. A serrated edge was cut into the bottom end of the paper so that solvent systems could flow off evenly. All phosphates were applied as ammonium salts. Three μl. of each organic phosphate (0·05M solution as free acid or ester), 2 μl. of orthophosphate (also 0·05M) and 8 – 10 μl. of the honey phosphate fractions were spotted, which produced spots of intensity comparable to the standards. Orthophosphate was used as a reference on all chromatograms. Solvent systems were allowed to run off the paper unless stated otherwise.

Chromogenic reagents

(*a*) For phosphate: molybdate reagent (Hanes & Isherwood, 1949) according to the procedure described by Subers *et al.* (1965).

(*b*) For carbohydrate: benzidine-citrate reagent according to White and Maher (1953) described by Porter and Hoban (1954) except that papers were dipped individually into the reagent. A chocolate-brown spot was produced by G-6-P, a yellow spot was slowly formed by G-1-P, and no colour was produced by the three-carbon phosphates.

(*c*) For amino groups: ninhydrin reagent (0·2% in acetone). Papers were sprayed twice, allowed to dry between sprayings, then heated at 100° C. for 1 minute. All the standard phosphorylated amino compounds listed produced purple spots.

RESULTS AND DISCUSSION

The honey phosphate fractions (Table 1) and all the standard phosphates were chromatographed together according to Procedure A (Table 2). Fraction 1 and 2 each produced two phosphate components, and Fraction 3 produced three phosphates. The absence of characteristic UV quenching and purple ninhydrin spots showed that the nucleotides and phosphorylated amino compounds were not present. However, ninhydrin did produce beige spots with the honey phosphates, standard phosphoglyceric acids and glycerophosphates. The fastest moving phosphate component in Fraction 3 migrated further than all the standard phosphates. The migration of the other honey phosphate components resembled that of the standard sugar phosphates and three-carbon phosphates.

The honey phosphate fractions, standard sugar phosphates and three-carbon phosphates were next chromatographed according to Procedure B (Table 2). In this situation, the honey phosphate components (except the fastest moving component in Fraction 3 whose travel was retarded by borate) were most similar to the glucose phosphates, Man-6-P and the three-carbon phosphates, because their migration was unchanged from that shown in Procedure A (Table 2). The migration of the fructose phosphates, ribose 5-phosphate and 6-phosphogluconate was retarded on the borate-impregnated paper (Procedure B); therefore, it was concluded that these phosphates were not present. The presence of Man-6-P is unlikely because of its toxicity to honeybees (Sols *et al.*, 1960). Preparative paper chromatography of Fractions 1 and 3 (Fig. 1) produced further resolution of their phosphate components. Two phosphate areas from each fraction appeared: 1*a* and 1*b* from Fraction 1; 3*a* and 3*b* from Fraction 3. Fraction 3*b* contained the phosphate which moved faster than any of the standard phosphates in Procedure A, and whose travel was retarded by borate in Procedure B. No further study of Fraction 3*b* was made at this time. Fraction 2 was not subjected to preparative paper chromatography because of its insufficient phosphate content.

Because neither Procedure A nor E (Fig. 2, Table 2) provided clear-cut separation of the glucose phosphates from some of the three-carbon phosphates, Procedure C (Table 2) was tried. Clear separation of the glucose phosphates from three-carbon phosphates occurred. The honey phosphates showed the same

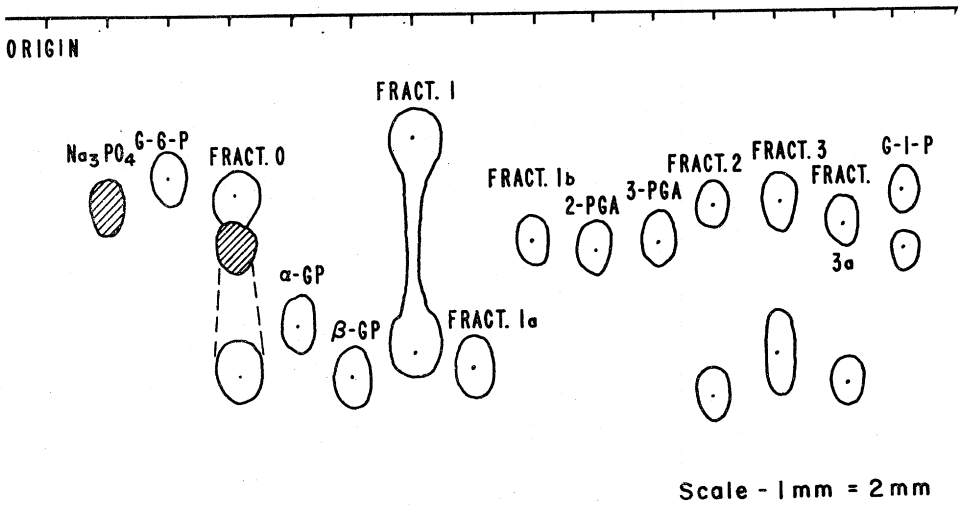

FIG. 2. A chart constructed from several paper chromatograms, showing the migration of the standard organic phosphates and the honey phosphates relative to the migration of orthophosphate (Procedure *E*, Table 2). Shaded spots indicate inorganic phosphate.

type of group separation, but the spots were not as well defined. With Procedure D (Table 2) the sugar phosphates separated from the three-carbon phosphates just as in Procedure C.

In addition, further resolution of the three-carbon phosphates occurred when α- and β-glycerophosphate migrated further than the other phosphates. Honey phosphates showed the same kind of resolution (Fig. 3), which indicated their similarity to G-6-P, phosphoglyceric acids and glycerophosphates. G-1-P produced double spots (Fig. 2, 3). The honey phosphates produced clearer, well defined spots in Procedure D. The migration and stability of the honey phosphates in Procedure E (Fig. 2) and Procedure F also suggested their similarity to G-6-P, phosphoglyceric acids and glycerophosphates. DHAP, G-3-P, 2,3-PGA, and PEP were all decomposed in Procedure F. PEP and 2,3-PGA were unstable in both Procedures E and F.

The similarity of the sugar phosphate in Fraction 0 (Figs. 1, 3) to G-6-P was also shown by the formation of a chocolate-brown spot with benzidine-citrate reagent. Although the position of the sugar phosphate spots in the silica gel column fractions (Figs. 1, 3) resembled that of G-6-P when these spots were detected by the molybdate reagent, no visible colour was obtained with the benzidine-citrate reagent. Because the benzidine-citrate reagent is not as sensitive as the molybdate reagent, the amount of carbohydrate material in the sugar phosphate was probably too small to be detected.

The stability and migration of the faster moving honey fractions (Figs. 2, 3) indicated the presence of phosphoglyceric acids and glycerophosphates. Because the migration of the 2- and 3-phosphoglyceric acids was so similar in all the procedures, it was impossible to determine which of these two isomers was present in the honey fractions, or whether both were present. The honey phosphate

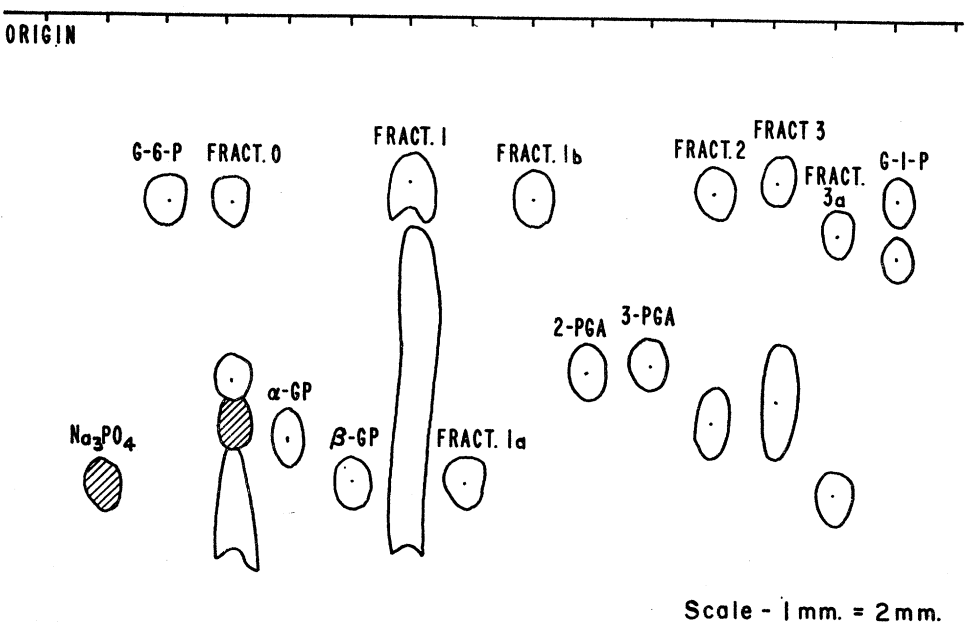

FIG. 3. A chart constructed from several paper chromatograms, showing the migration of the standard organic phosphates and the honey phosphates relative to the migration of orthophosphate (Procedure *D*, Table 2). Shaded spots indicate inorganic phosphate.

components, which migrated near the α- and β-glycerophosphates, did not show sufficiently reproducible movement to permit their specific identification as the α- or β-isomer. Free glucose and inorganic phosphate were found in Fraction 0 (Fig. 1). The free glucose may have come from hydrolysis during the removal of the phosphate fraction from the honey, or it may have remained in the fraction even after thorough washing of the ion exchange columns with water.

Three organic phosphates in honey have been partially separated and tentatively identified as glucose 6-phosphate, 2- or 3-phosphoglyceric acid and α- or β-glycerophosphate. An additional organic phosphate, which migrated further than any of the standard phosphates used in this investigation and whose travel was retarded by borate, was separated but not identified.

ACKNOWLEDGMENT

The authors wish to thank Dr. Jonathan W. White, Jr., for his suggestions and encouragement throughout this investigation.

REFERENCES

ALBON, N. & GROSS, D. (1952) The chromatographic determination of raffinose in raw sugars. Improvements in technique. *Analyst, Lond.* 77 : 410–412

AGARWAL, D. P., SANWAL, G. G. & KRISHNAN, P. S. (1962) A solvent system for the one-dimensional separation of hexose phosphates on filter-paper chromatograms. *Analyst, Lond.* 87 : 758 only

AGARWAL, D. P., SANWAL, G. G. & KRISHNAN, P. S. (1963) Improved paper-chromatographic separation of sugar phosphates by using borate-impregnated paper. *Analyst, Lond.* 88 : 969–970

BANDURSKI, R. S. & AXELROD, B. (1951) The chromatographic identification of some biologically important phosphate esters. *J. biol. Chem.* 193 : 405–410

BULEN, W. A., VARNER, J. E. & BURRELL, R. C. (1952) Separation of organic acids from plant tissues. Chromatographic technique. *Analyt. Chem.* 24 : 187–190

ERLENMEYER, E. & PLANTA, A. VON (1874) Ueber die Fermente in den Bienen im Bienenbrot und im Pollen und über einige Bestandtheile des Honigs. *S.B. bayer. Akad. Wiss.* 4 : 204–207

HANES, C. S. & ISHERWOOD, F. A. (1949) Separation of the phosphoric esters on the filter paper chromatogram. *Nature, Lond.* 164 : 1107–1112

MORTIMER, D. C. (1952) Paper chromatographic separation of some biologically important phosphate esters. *Can. J. Chem.* 30 : 653–660

PAUL, J. (1960) Simultaneous determination of silicon and phosphorus in the presence of each other. *Analytica chim. Acta* 23 : 178–182

PETTY, J. (1955) unpublished data

PORTER, W. L. & HOBAN, N. (1954) Ultramicrotechnique for enzymatic hydrolysis of sugars prior to chromatogram analysis. *Analyt. Chem.* 26 : 1846–1848

RUNECKLES, V. C. & KROTKOV, G. (1957) The separation of phosphate esters and other metabolites by ionophoresis and chromatography on paper. *Archs Biochem. Biophys.* 70 : 442–453

SCHUETTE, H. A. & HUENINK, D. J. (1937) Mineral constituents of honey II. Phosphorus, calcium, magnesium. *Fd. Res.* 2 : 529–538

SOLS, A., CADENAS, E. & ALVARADO, F. (1960) Enzymatic basis of mannose toxicity in honey bees. *Science, N.Y.* 131 : 297–298

STINSON, E. E., SUBERS, M. H., PETTY, J. & WHITE, J. W., JR. (1960) The composition of honey. V. Separation and identification of the organic acids. *Archs Biochem. Biophys.* 89 : 6–12

SUBERS, M. H., SCHEPARTZ, A. I. & KOOB, R. P. (1965) Submitted for publication

WHITE, J. W., JR. & MAHER, J. (1953) Transglucosidation by honey invertase. *Archs Biochem. Biophys.* 42 : 360–367

WHITE, J. W., JR. & PETTY, J. (1955) Unpublished data

BEE RESEARCH ASSOCIATION

The Bee Research Association is the central clearing house for information about research on bees and related subjects, including beekeeping and covering all species of bees.

The Association publishes three journals:

Journal of Apicultural Research, an international journal printed in English, for original research papers.

Apicultural Abstracts, a quarterly survey of scientific papers from world literature, in English.

Bee World, the official organ of the Association, which provides beekeeper and scientist with news and articles on recent developments.

Members are entitled to receive one or more of these journals. Membership has other advantages, including use of the Association's extensive Library of books, journals, reprints, translations and pictures, which are lent by post to any country in the world.

The journals can also be obtained by subscription.

Further details, including a complete list of B.R.A. publications, from:

BEE RESEARCH ASSOCIATION

Woodside House, Chalfont Heights, Gerrards Cross, Bucks., England